Combined Water Activity and Solute Effects on Growth and Survival of *Listeria monocytogenes* Scott A

ABSTRACT

Water activity and solute effects were determined on the growth and survival of *Listeria monocytogenes* Scott A in bacteriological media. Media were adjusted to water activity (a_w) levels of 0.99-0.80 with NaCl, glycerol (GLY), or propylene glycol (PG). Minimum a_w levels for growth occurred at 0.90, 0.92, and 0.97 for GLY, NaCl, and PG, respectively. Survival was related to the a_w level, as well as the solute employed. Cells survived the longest in GLY and died earliest in PG; NaCl was intermediate. These results provide quantitative isothermal criteria for estimating the a_w level necessary to prevent growth of *L. monocytogenes* in food.

Studies on the effect of water activity (a_w) on the growth and survival of the foodborne intracellular pathogen *Listeria monocytogenes* have been limited principally to the use of salt as a solute. Simon (*15*) was among the first to report *L. monocytogenes*' halotolerance. Seeliger (*13*) determined that the organism is capable of growing to 10% NaCl ($a_w \approx 0.93$). Others have demonstrated that temperature and salt combinations affected its growth. For example, Conner et al. (*5*) showed that *L. monocytogenes* Scott A and LCDC 81-861 grew in cabbage juice containing 2% NaCl ($a_w \approx 0.99$) but did not grow at 5% NaCl ($a_w \approx 0.97$) at 30°C. Yet, Benganovic et al. (*2*) reported that a strain of the organism which was isolated from sheep grew at 6°C in 5% NaCl. Petran and Zottola (*11,12*) studied the effect of other humectants on growth or inhibition of *L. monocytogenes*, including propylene glycol, lactose, fructose, galactose, and sucrose.

Knowledge of the effect of water activity on survival of the pathogen is similarly limited to NaCl studies. Seeliger (*13*) demonstrated that some strains tolerated 20% NaCl ($a_w \approx 0.86$) for short periods and remained viable after 1 year in 16% NaCl ($a_w \approx 0.90$) at pH 6.0. Beganovic et al. (*2*) reported that the organism died in 10% NaCl in 15-30 d, at a rate which was related to the inoculum size. Shahamat et al. (*14*) found that *L. monocytogenes* strain No. 18 (serotype 1/2a) remained viable at 37°C for 15 d in 10.5% NaCl, 10 d in 13% NaCl, and 5 d in 20-30% NaCl. Survival times more than doubled at 22°C. The organism survived longer than 100 d in 30.5% NaCl ($a_w \approx 0.80$) at 4°C.

There is a need to obtain a better understanding of the effect of a_w on the growth kinetics and survival of *L. monocytogenes*. The aim of this study, therefore, is to establish how a_w - as modulated by different solutes affects growth and survival of *L. monocytogenes* strain Scott A.

MATERIALS AND METHODS

Strain and maintenance conditions

Listeria monocytogenes Scott A, from the culture collection at the U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, was maintained at 4°C on brain heart infusion (BHI) agar streak plates and subcultured monthly. Culture purity and identity were confirmed at the time of each subculturing by Gram stain, catalase test, hemolytic activity on sheep red blood cell agar plates, and mobility test using phase contrast microscopy. For the experiments described here, a single colony was picked and grown overnight in 100 ml pH 7.4, BHI broth at 30°C at 300 rpm. One-ml portions of the culture were distributed into sterile glass vials and mixed with 20 µl dimethyl sulfoxide as a cryoprotectant. Vials were frozen immediately in dry ice and stored at -70°C. For each experiment a vial was removed, thawed at 4°C, and 10 µl transferred to 100 ml fresh BHI broth. The inoculum was grown overnight as described above. For growth/survival studies, the overnight culture was diluted to $\log_{10}$ 3-4 CFU/ml in 0.1% peptone (Difco Laboratories, Detroit, MI).

Experimental procedure

BHI broth (Difco Laboratories, Detroit, MI) containing 0.5% NaCl was adjusted to a_w levels of 0.99, 0.97, 0.93, 0.90, 0.87, 0.83, and 0.80 with NaCl (Mallinckrodt, Inc., Paris, KY), glycerol (GLY) from Sigma Chemical Co., St. Louis, MO or propylene glycol (PG) from Fisher Scientific Co., Fairlawn, NJ. Salt containing samples were prepared to a_w levels of 0.92 and 0.91, as well. All chemicals were used as received from the manufacturer or distributor. Solute levels for a_w modulations were obtained from Niven (*10*), Grover and Nicol (*7*), and Sloan and Labuza (*16*) for NaCl, GLY, and PG, respectively. Water activities were confirmed with a Rotronic Hygroskop DT water activity meter

(Rotronic Instrument Corp., Huntington, NY), equipped with a humidity sensor. Triplicate measurements were performed on each treatment combination.

One hundred ml of each medium in 250-ml flasks was adjusted to pH 7.4 with dilute HCl or NaOH, prior to sterilization. Flasks were prepared in triplicate and were frozen and stored at -18°C. Prior to each experiment, one of the three replicate flasks was thawed overnight at 4°C and a culture was prepared as described above. Experimental flasks were equilibrated to 28°C, inoculated with $\log_{10}$ 3-4 CFU/ml, and incubated at 28°C at 150 rpm. Samples were taken periodically, diluted serially with 0.1% peptone, and plated onto tryptose phosphate (TP) agar (Difco) using a Spiral Plater (Spiral Systems Inc., Cincinnati, OH). Petri dishes were incubated inverted for 48 h at 35°C and then enumerated using a Model 500 A Bacteria Colony Counter (Spiral Systems Instruments, Inc.). The threshold level of detection was 21 CFU/ml. Survival was defined as the ability of the organism to be enumerated under the conditions detailed above.

Curve fitting

Growth curves were fitted for solute and a_w treatments using the Gompertz equation (*3,6*). Gompertz coefficients were used to derive values of more practical use, including:

Exponential growth rate (EGR), $\log_{10}$ CFU/ml/h

Generation time (GT), h

Lag-phase duration (LPD), h, and

Maximum population density (MPD), $\log_{10}$ CFU/ml.

Where there was evidence of cell death, decimal reduction times (D_1 and D_2 values) were calculated using the biphasic logistic equation proposed by Kamau et al. (*8*) and modified by Dr. Richard C. Whiting (ERRC, Microbial Food Safety Research Unit) to include a lag factor (TL). The equation is:

$$\log N = \log\left[N_0\left[\frac{F_1(1+e^{-\beta_1 TL})}{(1+e^{\beta_1(t-TL)})} + \frac{(1-F_1)1+e^{-\beta_2 TL})}{(1+e^{\beta_2(t-TL)})}\right]\right]$$

where: N = surviving population (CFU) at time t; N_0 = initial population (CFU); F_1 = population fraction in major group; 1- F_1 = F_2 = population fraction in minor group; β_1 = constant for major group; β_2 = constant for minor group; TL = lag time, and *e* is the base of the natural logarithm. The D values were calculated by the equation $D_i = 2.3/\beta_i$.

RESULTS

Sodium chloride

Growth/inhibition curves are presented in Fig. 1 and results from the Gompertz curve fitting are presented in Table 1. Growth occurred at water activities of 0.99, 0.97, 0.93, and 0.92. EGRs decreased from 0.44 to 0.05 $\log_{10}$ CFU/h. Conversely, GT and LPD increased with decreases in a_w. MPD decreased with lowering of a_w levels from $\log_{10}$ CFU/ml 10.22 to 7.60, for a_w = 0.99 to 0.92, respectively. Below a_w levels of 0.92 the death rate (D_1) of *L. monocytogenes* Scott A was proportional to water activity; D values varied between 160 h for a_w = 0.91 to 28 h for a_w = 0.80. There was evidence of a shoulder (lag) that became less pronounced with an decreasing a_w. Duration of the estimated lag times (TL) before death ranged from 29 h (a_w = 0.80) to 278 h (a_w = 0.91). Survival of the organism in NaCl at 28°C was estimated to range from 200-700 h, depending upon water activity (Fig. 1). Studies using NaCl were conducted in greater detail than the remaining solutes because of its common use in foods.

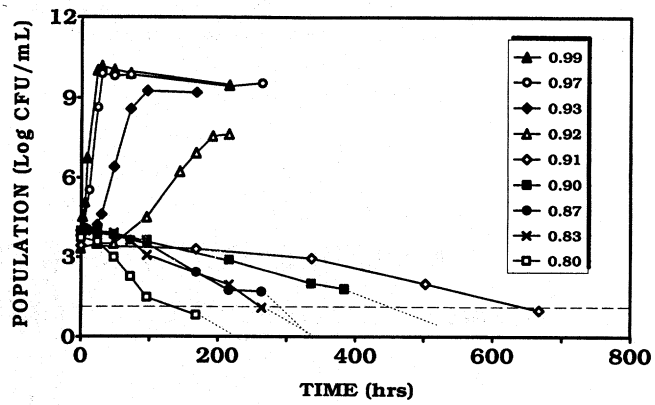

Figure 1. *Combined NaCl and a_w effects on growth and inhibition of* L. monocytogenes *Scott A in BHI medium at 28°C. Dotted lines are extrapolations of enumeration data. The minimum level of detection was 21 CFU/ml.*

Glycerol

The effect of GLY on growth or inhibition of *L. monocytogenes* is presented in Fig. 2 and Table 1. In contrast to NaCl, growth occurred in GLY to an a_w = 0.90. GT increased (0.8-17 h) over the permissive growth a_w range of 0.99 to 0.90, as did LPD (2-53 h); EGR decreased, from 0.4 to 0.02 $\log_{10}$ CFU/h. MPD decreased from $\log_{10}$ 10 CFU/ml for 0.99 to 8.5 at the minimum permissive a_w (0.90). The most significant difference between data from NaCl and GLY grown *L. monocytogenes* was a pattern of shorter lag time durations in GLY grown cells. At a_w levels of $\leq$ 0.87 there was no growth in a GLY containing medium. The death curves were generally parallel for a_w = 0.87-0.80. Differences observed in the shoulder (TL) or D value (D_1) did not show an obvious trend, in contrast to the NaCl data. Where death occurred at common a_w levels, the shoulders (TL) for the GLY treatments were uniformly greater than any of the NaCl treatments and *L. monocytogenes* survived longer in GLY than in NaCl. Survival in GLY was 400-500 h at 28°C (Fig. 2).

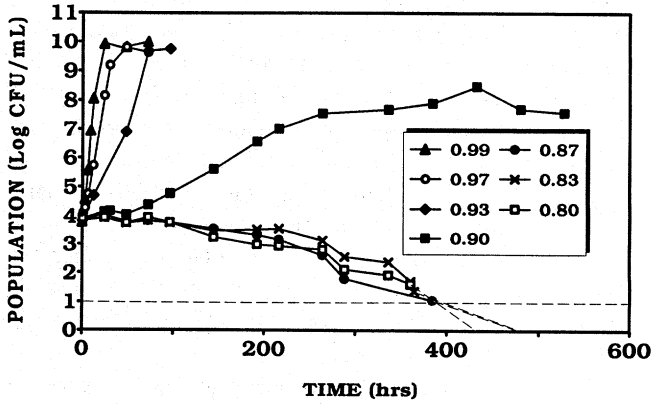

Figure 2. *Combined GLY and a_w effects on growth and inhibition of* L. monocytogenes *Scott A in BHI medium at 28°C. Dotted lines are extrapolations of enumeration data. The minimum level of detection was 21 CFU/ml.*

Propylene glycol

PG was the most toxic solute tested toward *L. monocytogenes* strain Scott A (Fig. 3 and Table 1). Growth occurred only at $a_w \geq 0.97$. Where conditions were permissive for growth in the presence of PG, EGR, GT, and LPD

TABLE 1. *Growth/Survival kinetics of* L. monocytogenes *Scott A in various solutes and water activities (a_w).*

Solute	Gompertz derivatives					D-Value parameters						
	a_w	EGR[a]	GT	LPD	MPD	TL	D1	F2	D2	SS[b]	RMS	N
Salt	0.99	0.44	0.69	3.04	10.22					0.12	0.13	7
	0.97	0.35	0.86	6.90	9.84					0.31	0.20	8
	0.93	0.12	2.55	27.20	9.55					0.01	0.04	7
	0.92	0.05	6.40	68.40	7.60					0.15	0.13	9
	0.91					278	159.9			0.00	0.03	5
	0.90					108	118.7			0.13	0.10	12
	0.87					63	71.3			0.31	0.19	9
	0.83					75	60.0			0.60	0.26	9
	0.80					29	27.7			0.17	0.14	8
Glycerol	0.99	0.40	0.76	2.11	10.09					0.28	0.19	7
	0.97	0.24	1.28	3.67	9.92					0.25	0.16	8
	0.93	0.12	2.52	7.61	9.74					0.26	0.19	7
	0.90	0.02	16.64	52.75	8.48					0.35	0.17	12
	0.87					138	84.5			0.25	0.15	11
	0.83					212	69.0			0.33	0.16	13
	0.80					115	106.1			0.17	0.12	12
Propylene glycol	0.99	0.41	0.73	2.58	9.90					0.34	0.21	8
	0.97	0.24	1.26	3.10	9.10					0.18	0.13	10
	0.93					15	123.1			0.21	0.15	9
	0.90					98	43.9			0.25	0.17	9
	0.87					15	13.5	-2.3	153.4	0.09	0.11	8
	0.83					4	8.3	-1.9	104.7	0.07	0.11	6
	0.80					8	3.5	-1.8	90.5	0.09	0.14	5

[a] See text for abbreviations related to Gompertz and biphasic logistic equation.
[b] SS: sum of squares; RMS: root mean square; N: number of samples.

were similar to GLY grown *L. monocytogenes*. Compared to NaCl grown *L. monocytogenes* at a_w = 0.97, however, PG grown cells exhibited slower EGR (0.4 vs 0.2 $\log_{10}$ CFU/h), longer GT (0.86 vs 1.26 h), shorter LPD (7 vs 3 h), and lower MPD (9.84 vs 9.10 $\log_{10}$ CFU/ml).

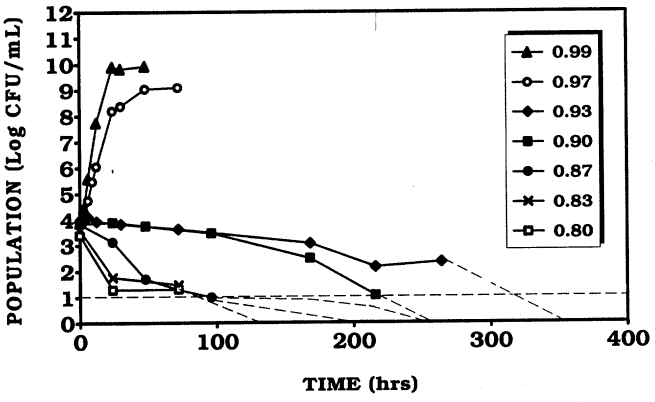

Figure 3. *Combined PG and a_w effects on growth and inhibition of* L. monocytogenes *Scott A in BHI medium at 28°C. Dotted lines are extrapolations of enumeration data. The minimum level of detection was 21 CFU/ml.*

Cells grown in PG died faster as a_w levels decreased. At a_w levels of 0.93 and 0.90, the survival and death of the cells exhibited a shoulder (TL), followed by a first order decline (D_1). Values for TL and D_1 were 15 and 123 h and 98 and 44 h, for a_w = 0.93 and a_w = 0.90, respectively. Both other humectants permitted growth at a_w = 0.93. At a_w = 0.90, NaCl grown cells exhibited longer lag times (108 vs 98 h) and longer D_1 values (118 vs 44 h). Unlike NaCl and GLY, at PG modulated a_w levels between 0.87 and 0.80, the *L. monocytogenes* survival and death curves appeared to be biphasic and were best fitted using two D values. At these a_w levels the D_1 values were small, being 12, 7, and 3 h, for a_w = 0.87, 0.83, and 0.80, respectively. The D_2 values were: 183, 104, and 91 h for a_w = 0.87, 0.83, and 0.80, respectively. The F_2 values expressed in Table 1 are the $\log_{10}$ surviving fraction, which became larger with increases in the D_2 value.

DISCUSSION

Christian (*4*) suggested that at least three solutes should be compared when studying solute effects on the minimum a_w permitting growth of microorganisms. Therefore, in the present study a comparison of NaCl, GLY, and PG was conducted to determine water activity effects on the growth and inhibition of *L. monocytogenes* strain Scott A. The observed growth in NaCl of *L. monocytogenes* to an a_w = 0.92 (11.5%) at 28°C is slightly lower than the growth observed at 10% by Seeliger (*13*). Differences in incubation temperature, however, may account for the disparity, since these temperatures and water activity act synergistically. Growth in PG occurred at a_w = 0.97 (16.7%, w/w), but not below, indicating that this solute is more limiting than NaCl at an equivalent a_w. Petran and Zottola (*11*) observed that *L. monocytogenes* Jalisco cheese grew in 10%, but not 20% PG in 0.1% nonfat dried milk at 4°C,

which is consistent with the current study. The inhibitory effect of this polyol has been noted for many microorganisms (*1*). Growth in glycerol was observed in the current study to a_w = 0.90, the lowest water activity of the three solutes tested.

The effect of decreased a_w levels on growth of microorganisms was described by Troller (*18*) as: an extension of lag phase, a suppression of the log phase, and a reduction in the total number of viable microorganisms. The data from the three solutes studied in the present investigation are generally in agreement with this statement. The current study also demonstrated that at identical a_w levels *L. monocytogenes* growth behavior varied with the solute employed.

Survival in BHI media adjusted to a_w levels between 0.99 to 0.80 was also affected by the different solutes. Generally, the patterns for survival were identical to growth: longest survival in GLY and shortest in PG, with NaCl intermediate, at equal a_w levels. Regarding survival in NaCl, Shahamat et al. (*14*) found similar results as the current study. These investigators also observed that decreasing temperature permitted longer survival at equal NaCl levels. Survival studies in GLY or PG appear to be unique to the current research.

Solute effects, with respect to water activity, on the growth of foodborne pathogens have been observed by other investigators. Marshall et al. (*9*) showed that rods were more sensitive to NaCl than to glycerol. Similar conclusions were reached by Sperber (*17*). Webster et al. (*19*) suggested that in general ionic solutes are more inhibitory than low molecular weight solutes. Webster et al. (*19*) also concluded that at reduced a_w, inhibition may be lower for permeating solutes such as glycerol than for relatively impermeable solutes such as sodium chloride.

The mechanism of the interaction of solutes and a_w effects on *L. monocytogenes* may be explained by theoretical considerations of the control of bacterial growth by a_w (*18*). Troller's review of the literature indicated that by lowering a_w levels, solutes cause water loss through plasmolysis and disruption of intracellular K^+ homeostasis. Loss of positive turgor pressure within the cell results. The high permeability of solutes such as glycerol across the bacterial plasma membrane may have only a transitory effect on cell turgor, unlike a plasmolyzing solute such as NaCl (*4*).

The use of equations for modeling microbial growth permits a quantitative comparison of the effects of treatments on growth kinetics. Although empirical, the Gompertz equation has been used frequently for these purposes (*3,6*). Regarding survivor curves, the classic equations for thermal inactivation generate D, z, and F values, which assume linearity of the decline. The results shown here and by others (*8*) demonstrate the curvilinear nature of nonthermal killing curves. For the present study some of the inactivation curves required a lag factor (TL) to account for the delay or shoulder before killing was apparent. To this end the biphasic logistic equation was modified.

The data from Table 1 indicate that values for most of the Gompertz derivative and D-value parameters change more rapidly as a_w decreases, regardless of solute or growth or survival curves. This suggests that the modulating effect of water activity on growth or survival is exponential or second order. The D_2 for PG is an exception.

While speculative, the apparent biphasic death curve observed in the PG treated cells suggests that a substantially large percentage of the population was killed relatively fast, leaving behind a second, more resistant subpopulation. An alternative hypothesis is that microorganisms were inactivated by dual inactivation mechanisms.

The observation that some parameters listed in Table 1 exhibited marked rates of change as water activity decreased suggests an exponential or second order relationship. This has implications for water activity modulation as a significant growth controlling variable in foods.

The findings presented in the current study are applicable for use with the barrier concept for pathogen inhibition in foods, whereby multiple sublethal factors work synergistically to minimize microbiological safety risks. Such barriers include refrigeration, pH, water activity, and antimicrobial agents. Many foods now use multiple barriers to maintain safety. Thus, if a_w is an intended growth barrier, the target level must be appropriate for the solute modifying equilibrium relative humidity. Water activities at interfaces and in other microenvironments, as well as the interactive effects of multiple variables on pathogen growth, must be also considered in the design considerations of safe foods.

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REFERENCES

1. Barr, M., and L. F. Tice. 1957. A study of the inhibitory concentrations of glycerol-sorbitol and propylene glycol-sorbitol combinations on the growth of microorganisms. J. Am. Pharm. Assoc. 46:217-218.
2. Benganovic, A. H., A. Milanovic, and Z. Forsek. 1971. The viability of *Listeria monocytogenes* in NaCl solution and pickle. Veterinaria 20:73-80.
3. Buchanan, R. L., and J. G. Phillips. 1990. Response surface model for predicting the effects of temperature, pH, sodium chloride content, sodium nitrite concentration, and atmosphere on the growth of *Listeria monocytogenes*. J. Food Prot. 53:370-376.
4. Christian, J. H. B. 1981. Specific solute effects on microbial water relations. pp. 825-854. *In* L. B. Rockland and G. F. Steward (ed.), Water activity: Influences on food quality. Academic Press, New York.
5. Conner, D. E., R. E. Brackett, and L. R. Beuchat. 1986. Effect of temperature, sodium chloride, and pH on growth of *Listeria monocytogenes* in cabbage juice. Appl. Environ. Microbiol. 52(1):59-63.
6. Gibson, A. M., N. Bratchell, and T. A. Roberts. 1988. Predicting microbial growth: growth responses of salmonellae in a laboratory medium as affected by pH, sodium chloride, and storage temperature. Int. J. Food Microbiol. 6:155-178.
7. Grover, D. W., and J. M. Nicol. 1940. The vapor pressure of glycerin solutions at 20°C. J. Soc. Chem. Ind. 59:175-177.
8. Kamau, D. N., S. Doores, and K. M. Pruitt. 1990. Enhanced thermal destruction of *Listeria monocytogenes* and *Staphylococcus aureus* by the lactoperoxidase system. Appl. Environ. Microbiol. 56:2711-2716.
9. Marshall, B. J., D. F. Ohye, and J. M. B. Christian. 1971. Tolerance of bacteria to high concentrations of sodium chloride and glycerol in the growth medium. Appl. Microbiol. 21:363-364.

10. Niven, C. F. 1987. Microbiology and parasitology of meat. pp. 217-263. *In* J. F. Price and B. S. Schweigert (ed.), The science of meat and meat products, 3rd Ed. Food & Nutrition Press, Westport, CT.
11. Petran, R., and E. A. Zottola. 1988. Survival of *Listeria monocytogenes* in simulated milk cooling systems. J. Food Prot. 51:172-175.
12. Petran, R., and E. A. Zottola. 1989. A study of factors affecting growth and recovery of *Listeria monocytogenes* Scott A. J. Food Sci. 54:458-460.
13. Seeliger, H. P. R. 1961. Listeriosis. Hafner, New York.
14. Shahamat, M., A. Seaman, and M. Woodbine. 1980. Survival of *Listeria monocytogenes* in high salt concentrations. Zentrabl. Bakeriol. Hyg. I. Abt. Orig. A246:506-511.
15. Simon, C. 1955. Möglichkeiten zur Anreicherung von *Listeria monocytogenes* in flüssigen Vorkulturen. Z. Hyg. InfektKrankh. 143:159-172.
16. Sloan, A. E., and T. P. Labuza. 1975. Investigating alternative humectants for use in foods. Food Prod. Dev. (9):75.
17. Sperber, W. H. 1983. Influence of water activity on food borne bacteria. J. Food Prot. 46:142-150.
18. Troller, J. A. 1989. Water activity and food quality. pp. 1-31. *In* T. M. Hartman (ed.), Water and food quality. Elsevier Science Publishing Co., New York.
19. Webster, S. N., D. R. Fowler, and R. D. Cooke. 1985. Control of a range of food related microorganisms by a multi-parameter preservation system. J. Food Technol. 20:311-318.